Luteal regression vs. prepartum luteolysis: Regulatory mechanisms governing canine corpus luteum function

Kowalewski, Mariusz P

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Corresponding Author: Dr. Mariusz Pawel Kowalewski, PD, DVM, PhD

Corresponding Author's Institution: University of Zurich

First Author: Mariusz Pawel Kowalewski, PD, DVM, PhD

Order of Authors: Mariusz Pawel Kowalewski, PD, DVM, PhD

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Dear Prof. Ciereszko,

Herewith, I would like to submit the revised final version of my manuscript for publication in Reproductive Biology.

Sincerely yours,

PD Dr. Mariusz P. Kowalewski, PhD

Institute of Veterinary Anatomy
Vetsuisse-Faculty
University of Zurich
Winterthurerstrasse 260
CH-8057 Zurich, Switzerland

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Kowalewski Mariusz Pawel

Institute of Veterinary Anatomy, Vetsuisse Faculty, University of Zurich, CH-8057 Zurich, Switzerland.

Correspondence:

PD Mariusz P. Kowalewski, PhD

Institute of Veterinary Anatomy

Vetsuisse Faculty

University of Zurich

Winterthurerstrasse 260

CH-8057 Zurich, Switzerland

Tel.: 0041-44-6358784

Fax.: 0041-44-6358943

Email: kowalewskipl@yahoo.de or kowalewski@vetanat.uzh.ch

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Kowalewski Mariusz Pawel

Institute of Veterinary Anatomy, Vetsuisse Faculty, University of Zurich, CH-8057 Zurich, Switzerland.

Correspondence:

PD Mariusz P. Kowalewski, PhD

Institute of Veterinary Anatomy

Vetsuisse Faculty

University of Zurich

Winterthurerstrasse 260

CH-8057 Zurich, Switzerland

Tel.: 0041-44-6358784

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Canine reproductive physiology exhibits several unusual features. Among the most interesting of these are the lack of an acute luteolytic mechanism, coinciding with the apparent luteal independency of a uterine luteolysin in absence of pregnancy, contrasting with the acute prepartum luteolysis observed in pregnant animals. These features indicate the existence of mechanisms different from those in other species for regulating the extended luteal regression observed in non-pregnant dogs, and the actively regulated termination of luteal function observed prepartum as a prerequisite for parturition. Nevertheless, the supply of progesterone (P4) depends on corpora lutea (CL) as its primary source in both conditions, resulting in P4 levels that are similar in pregnant and non-pregnant bitches during almost the entire luteal life span prior to the prepartum luteolysis. Consequently, the duration of the prolonged luteal phase in non-pregnant bitches frequently exceeds that of pregnant ones, which is a peculiarity when compared with other domestic animal species. Both LH and prolactin (PRL) are endocrine luteotrophic factors in the dog, the latter being the predominant one. In spite of increased availability of these hormones, luteal regression/luteolysis still takes place. Recently, possible mechanisms regulating the expression and function of PRL receptor have been implicated in the local, i.e., intraluteal regulation of PRL bioavailability and thus its steroidogenic potential. Similar mechanisms may relate to the luteal LH receptor. Most recently, evidence has been provided for an autocrine/paracrine role of prostaglandin E2 (PGE2) as a luteotrophic factor in the canine CL acting at the level of steroidogenic acute regulatory (STAR)-protein mediated supply of steroidogenic substrate, without having a significant impact on the enzymatic activity of the respective steroidogenic enzymes, 3β-hydroxysteroid-dehydrogenase (3βHSD, HSD3B2) and cytochrome P450 side-chain cleavage enzyme (P450scc, CYP11A1). Together with the strongly time-dependent expression of prostaglandin transporter, luteal prostaglandins seem to be involved more in the process of luteal formation than in termination of CL function in the dog. The possible roles of other factors such as vasoactive compounds, growth factors or cytokines have not been extensively studied but should not be neglected.

1. Introduction

As the primary source of progesterone (P4) during pregnancy and the non-pregnant cycle, corpora lutea seem to be key organs regulating the reproductive cycle in dogs. Hence, not surprisingly, the physiological mechanisms regulating CL function have been subjected to increased scientific interest. However, even though expression and/or function of several,

mostly luteotrophic, factors have been characterized, overall understanding of canine CL physiology remains poor, especially during its regression. Dogs are highly important in veterinary medicine, have a substantial role as laboratory animals and are also among the most important pets. Taking all of this into account, efforts need to be made to better understand canine reproductive physiology, especially that concerning CL function.

In addition to the lately debate (e.g., [1-6]), here an overview of our current knowledge concerning the endocrine, paracrine and autocrine control of the CL in non-pregnant and pregnant dogs, together with some new, unpublished data recently generated in our laboratory, e.g., concerning the expression of 15-hydroxy prostaglandin dehydrogenase (HPGD) and the LH receptor (LHR), is presented.

2. Luteal life span and patterns of steroid secretion

Among domestic animal species, the domestic dog (*Canis lupus familiaris*) is the only one classified as a predominantly non-seasonal, monoestrus breeder. The sexual cycle is characterized by a long luteal phase (diestrus) and an obligatory sexual quiescence phase between sexually active periods (anestrus), the length of which may depend on the breed [7]. Unlike in livestock, in which cyclicity depends on periodic production and secretion of luteolytic prostaglandin F2α (PGF2α) by the uterus, at least in non pregnant dogs the luteal function is independent of a uterine luteolysin, because ovarian cyclicity is maintained following hysterectomy [8]. Consequently, also pointing towards inherent control mechanisms, the physiological luteal lifespan is similar in pregnant and non-pregnant bitches.

2.1. Progesterone

Following preovulatory luteinization, peripheral progesterone (P4) reaches levels of about 5 ng/ml at the time of ovulation [9]. After ovulation, strongly increasing luteal steroidogenic capacity results in the highest circulating P4 levels 15-25 days, or even up to 30 days after ovulation [8]. Then, a progressive P4 decrease marks the onset of luteal regression, which lasts in non-pregnant dogs as long as 1-3 months, until peripheral P4 levels falling below 1 ng/ml, by definition, indicate the onset of anestrus [9]. The P4 secretion pattern, which is up to that time-point similar in the non-pregnant and pregnant bitch, starts to differ at approximately day 60 of the luteal lifespan, when the circulating P4 concentration falls precipitously in the pregnant animal as a prerequisite for parturition (prepartum luteolysis), therefore rapidly reaching baseline levels.

While individual serum P4 levels vary strongly, the mean values can measurably differ between pregnant and non-pregnant bitches during the entire course of diestrus displaying values that are numerically but not statistically lower in the non-pregnant animals. In one study by Steinetz et al. [10], the mean P4 concentrations in pseudopregnant bitches were only 56% of the concentrations in pregnant females. Nevertheless, mostly due to the above-mentioned high individual variations, measurement of serum P4 is precluded as a means to distinguish between pregnant and non-pregnant dogs.

2.2. Estrogens

Because the canine placenta is devoid of steroidogenic activity [11,12], circulating estrogens appear to originate in corpora lutea (CL). This notion has been supported by studying the time-dependent expression of aromatase in canine luteal samples throughout diestrus [1,12]. There is, however, no pregnancy-associated increase in estradiol-17β (E2) production, and similar E2 secretion patterns are observed in both pregnancy and diestrus of non-pregnant dogs until the prepartum drop is observed, as for P4 [11,13]. This prepartum drop in E2 further implicates CL as the major source of estrogens in the dog.

Both E2 and P4 exert autocrine and/or paracrine, presumably luteotrophic, effects on the canine CL, since their respective receptors, i.e., P4 receptor (PGR) and estrogen receptors -alpha and -beta (ERα and ERβ), are expressed locally. This concept is further supported by the observation that interfering with P4 action at the level of PGR, e.g., by application of an antigestagen, results in preterm luteolysis [14,15]. It is possible that, indirectly, estrogens could act by priming PGR and prolactin receptor (PRLR) expression.

2.3. Cortisol

The elevated levels of cortisol measured in maternal blood peripartum appear to have erratic nature and seem to be not mandatory for normal parturition [16]. It has also been related to maternal stress [11, 16]. On the other hand, as suggested by Concannon and collaborators [16], it is possible that cortisol circulating in maternal blood prepartum merely reflects much larger increases at the feto-placental and uterine levels.

3. Luteal development, morphological and functional characteristics

As in other species, the formation of the canine CL starts from residual follicular cells right after ovulation. Early luteal development is characterized by strong proliferation of all cellular components, as indicated by increased expression of the Ki67 marker in lutein and non-lutein

cells [1]. At the same time the significantly increased staining of endoglin, points towards a high vascularization rate during the early luteal phase, especially on day 15 after ovulation [17], after which it is considerably lower. The strong vasculogenic and angiogenic activities are also evidenced by the expression of members of the vascular endothelial growth factor (VEGF) system in steroidogenic and non-steroidogenic cells [18].

Unlike in livestock, in which lutein cells can be divided into small- (theca interna-derived), and large- (granulosa cell-derived) lutein cells, no such distinction can be made in dogs. Although, as in other species, likewise, canine lutein cells develop from both of these cell populations, only one type of steroidogenic cell can be identified in dogs both histomorphologically and ultrastructurally [19] (Fig 1). The extent to which granulosa and theca cells contribute to CL formation is not known. However, the strong preovulatory luteinization observed in maturing canine follicles suggests an important role of granulosa cells in this process.

In the growing canine CL, the lutein cells are irregularly shaped, with a diameter at day 5 after ovulation of 5-10 μm, exhibiting large, bright nuclei containing one or two nucleoli and cytoplasm filled with small lipid droplets [19]. While extravasated erythrocytes can still be observed in luteal tissue, e.g., at days 5-10 after ovulation, indicating the haemorrhagic stage of development (Fig. 1A,B), already at this stage of luteal development intense vascularization is evident as a well-established capillary network. At day 15 after ovulation, lutein cells are approximately 20 μm in size, while the diameter of fully developed lutein cells at days 20-25 after ovulation is around 30-40 μm. They are polyhedral, tightly packed, with homogeneous nuclei, fewer lipid droplets than during the growth phase, but with large number of mitochondria and smooth endoplasmatic reticulum (Fig. 1C,D). At this time, in the mature CL, proliferative activity is already reduced [1] and the luteal vascular bed is fully established (Fig. 1C,D) providing virtually all lutein cells contact with a capillary [17]. The latter is a phenomenon known from other species, allowing for maximal supply of P4 from the CL to target organs, e.g., the utero/placental compartment. It is conceivable that the function of the freshly formed capillaries as well as of the lutein cells is modulated by a set of vasoactive factors that are as yet not fully defined in the dog.

The strong proliferative activity observed during early development of the CL is followed by continuously increasing steroidogenic activity, which in turn is reflected in the highly upregulated expression of steroidogenic acute regulatory protein (STAR) and 3β-hydroxysteroid-dehydrogenase (3βHSD, HSD3B2), the two key factors involved in the precise control of hormonal output from most of the steroidogenic tissues. STAR is

responsible for the active transport of cholesterol from the outer to the inner mitochondrial membrane and, hence, for the supply of steroidogenic substrate, whereas 3βHSD controls the last step of P4 synthesis, namely the conversion of pregnenolone to P4. The expression patterns of both factors throughout diestrus closely parallel the peripheral P4 content in pregnant and non-pregnant dogs [14,20,21]. Taking this into account, as well as the potentially superior role that STAR plays compared to 3βHSD (being responsible for the substrate supply for the subsequent enzymatic reaction), STAR-dependent mechanisms seem to play a rate-limiting role within the canine CL controlling luteal enzymatic conversion rate and, thus, steroidogenic capacity. This is further supported by a recent study from our group in which STAR, but not 3βHSD and cytochrome P450 side-chain cleavage enzyme (P450scc, CYP11A1; factor responsible for enzymatic cleavage of cholesterol to pregnenolone), was significantly affected in prostaglandin E2 (PGE2)-treated canine luteal cells isolated from early CL. This treatment resulted in increased STAR mRNA and protein expression and its functional activation, i.e., phosphorylation, as evidenced from up-regulated [2] steroidogenesis, which was similar to levels obtained with cAMP.

The cAMP/PKA pathway, involving activation of many transcriptional factors serving both in the basal and induced STAR-promoter activity, is indisputably the major pathway in the trophic-hormone dependent expression and activation of STAR. Recently, canine proximal -255/-1 promoter was cloned and compared with its well-characterized murine counterpart, revealing several putative binding sites for transcriptional factors, e.g., CCAAT-enhancer-binding protein (C/EBP), steroidogenic factor (SF)1/2, SF1/3, GATA, sterol regulatory element-binding protein (SREBP) or DAX1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1) [2]. Additionally, two cAMP-responsive element half-sites (CRE-half-sites) corresponding to murine CRE-1 and CRE-3, were found [2]. Finally, the functional responsiveness of canine STAR-promoter to cAMP was confirmed [2]. More detailed studies should be considered to elucidate the identity of the upstream regulating factors, as well as the relative importance of the specific transcriptional factors in STAR-mediated luteal progesterone synthesis in dogs.

As the CL enters the mid-luteal phase, STAR expression decreases [14,20] resulting in declining peripheral P4 levels; i.e., the luteal regression sets in. By day 30 after ovulation, the endoplasmic reticulum exhibits whirl-like structures and is localized mostly in the periphery of lutein cells, not close to the nucleus. Clear signs of structural regression become visible at day 45 after ovulation (Fig. 1E,F): intercellular distances become larger, the density of

capillaries decreases, the numbers of collagen fibres and cellular components of connective tissue increase, and larger lipid droplets start to be visible all over the cytoplasm of lutein cells, indicating ongoing fatty degeneration. The whirl-like smooth endoplasmic reticulum of steroidogenic cells encircles now large lipid droplets as a further sign of fatty degeneration [19], likely additionally contributing to diminished steroid production. Marked signs of degeneration can be seen approximately 20 days later (Fig. 1G,H). Lutein cells become irregular in shape and size, and chromatin condensation commences; somewhat later, nuclei become noticeably pyknotic, large lipid vacuoles are observed in the cytoplasm, the number of mitochondria decreases significantly, and the amount of connective tissue elements and collagen fibres increases significantly [19].

It is noteworthy that the above-described progressive luteal regression takes place absent of any active luteolytic principle, e.g., strong apoptotic signals, that would be observed in the CL of non-pregnant dogs, making apoptosis the major mechanism regulating functional luteal regression [6,19] in this species. Caspase-3 seems to be involved here in functional and structural regression and in the tissue reorganization associated with corpus albicans formation [22]. This is in contrast to pregnant dogs in which the steep P4 decline during prepartum luteolysis, coinciding with significantly increased levels of PGF2α in maternal peripheral blood, is associated with strong apoptotic signals, as evidenced by immunohistochemical staining for active caspase-3 (Fig. 2). This needs to be seen as indicative of different regulatory mechanisms involved in termination of CL function in pregnant vs. non-pregnant dogs, pointing towards an actively regulated process of CL destruction at the end of gestation. The immunohistochemical results presented in Fig. 2 were obtained according to our previously published protocol [21,23]. The primary antibody was rabbit anti-active caspase-3 IgG (BD Biosciences) at a dilution of 1:50; as a negative/isotype control, IgG irrelevant antibodies I-5000 (Vector Laboratories Inc., Burlingame, CA, USA) were used at the same concentration as the primary antibody; the secondary antibody was biotinylated goat anti-rabbit IgG BA-1000 from Vector Laboratories.

4. Immune system

Even though still not fully elaborated, there is evidence for potential involvement of the immune system in the regulation of canine luteal function. Thus, not surprisingly, the presence of CD4- and CD8- positive immune cells, as well as the expression of the major histocompatibility complex class II (MHC II)-complex, was confirmed in canine CL throughout the luteal life span of non-pregnant dogs. The cluster of differentiation (CD)4-

positive cells were identified as lymphocytes and macrophages, the CD8- positive cells were T-lymphocytes. The MHC II-complex stained for T-lymphocytes and macrophages and also for fibroblasts and some of the lutein cells [6,17]. Strong vascularization and early luteal development were characterized by increased presence of T-lymphocytes and macrophages. A renewed increase in CD8- and MHC II- positive immune cells was noticed late during luteal regression (at days 45- and 60 after ovulation, respectively), implying that, as in other species, in the dog, immune cells together with cytokines produced by them may be involved in regulatory processes at both ends of the luteal life span, i.e., at luteal formation and regression [6,17].

Only sparse information is available concerning luteal cytokines in the dog. The expression of interleukin (IL)-8, IL-10, IL-12, tumor necrosis factor (TNF)α and transforming growth factor beta 1 (TGFβ1), but not of IL1β, IL-2 and IL-4, could be detected qualitatively at the mRNA level [24]. The expression of IL-6 and interferon (IFN)γ was erratic and frequently below the detection limit [24]. Recently, in our ongoing project, the luteal expression of TNFα and its two receptors (TNFR-1 and -2) was confirmed at the protein level throughout diestrus *(own data, unpublished)*. Cumulatively, it may be reasonably assumed that immune system-derived cells and their products play an important or at least a modulatory role during the course of canine diestrus, with a functional shift between the beginning and the end of the CL phase. The involvement and role of individual factors remain, however, to be elucidated.

5. Gonadotrophic regulation

5.1. Secretion patterns of prolactin (PRL) and luteinizing hormone (LH)

After observing relatively low levels of serum PRL at the beginning of pregnancy, a substantial increase can be measured during the second half of gestation; after approximately 5-6 weeks, PRL levels start to increase steadily towards parturition [25-27], peaking at about 50 ng/ml near term [25]. In non-pregnant bitches, basal PRL remains low during most of the luteal phase, and increase 2-3 fold from the initial levels of 2-4 ng/ml, reaching maximum levels of about 9 ng/ml around the time corresponding to parturition in pregnant bitches [25,26]. High plasma PRL concentrations can also be measured in overtly pseudopregnant bitches, therefore, increased levels of PRL are not reliable indicators for detection of pregnancy [28]. The pregnancy-associated increase in PRL is concomitant with placental relaxin secretion that begins between days 25 and 30 and persists until term [29], suggesting a

stimulatory role of relaxin on PRL secretion, and possibly explaining higher P4 levels observed during pregnancy. Such a regulatory mechanism would resemble the modulatory role of relaxin on PRL secretion that was previously demonstrated in pigs and monkeys [30,31]. Within the canine placenta, the syncytiotrophoblast has been identified as the possible source of relaxin [32].

Similar to PRL, the concentrations of LH also appear to increase during the progression of diestrus. Thus, increased circulating LH has been observed while P4 concentrations continue to decrease, indicating that the cessation of luteal function does not result from a lack of either LH or PRL [33,34].

5.2. Effects of hypophysectomy

Whereas, indisputably, both PRL and LH are luteotrophic hormones in the dog, the extent to which pituitary support is required for luteal maintenance, as well as its exact timing, awaken some controversies and are the subjects of continuous debate in canine literature (see, e.g., [35-38]). Thus, during the first 2-4 weeks of luteal development, dynamically growing canine CL, producing increasing levels of P4, reveal a reduced sensitivity to luteolytic insults that are clearly effective later in diestrus. In this regard, as shown by Okkens and collaborators [39], hypophysectomy, and hence deprivation of gonadotrophic support, at day 4 after ovulation resulted only in a transient suppression in circulating P4 levels, which recovered to normal values within 6-10 days. This was, however, not the case for dogs hypophysectomized on day 18 of the luteal phase, whose P4 decline was permanent [39]. The initial acute P4 decrease observed after hypophysectomy performed at day 4 after ovulation was interpreted as a consequence of surgical stress, as indicated also elsewhere [40,41]. Interestingly, the luteal life span was significantly shortened in dogs in which P4 levels recovered, compared with controls. This can be seen as an indicative of the luteotrophic function of pituitary hormones predominantly during the later luteal stages. On the other hand, in studies performed by Concannon et al. [35], hypophysectomies performed on days 10-50 of the cycle resulted in permanent P4 suppression within 3-17 days.

Cumulatively, there is evidence indicating that the canine CL is to some extent autonomous, at least during its earliest developmental stages. Based on their investigations Okkens et al. [39] speculated that this period ends at days 24-28 of the CL life span. However, Concannon [35] postulated the canine CL to be chronically dependent on gonadotrophic support throughout the luteal phase. There is only low PRL gene expression in the canine CL and uterus throughout diestrus mostly below the detection limit. Therefore, even if a possible

auto/paracrine function of intraluteally secreted PRL cannot be ruled out, these do not seem to contribute significantly to the circulating PRL *(own data, unpublished).*

5.3. Luteotrophic function of PRL and LH

Whereas during the second half of its life span the canine CL depends upon gonadotrophic support, the deprivation of which unequivocally results in cessation of luteal function, some refractoriness can be observed during earlier luteal stages. Thus, suppression of PRL secretion by the dopamine agonist bromocriptine used over six days starting on days 8 and 22 of pregnancy resulted in only a temporal P4 decline during the treatment. This was followed by a 2-6 day recovery phase after which P4 concentrations returned to normal values and all dogs gave birth to normal litters [36]. In contrast, in the same study, in dogs treated on day 42 of pregnancy and on the corresponding day 42 of the non-pregnant cycle, P4 concentrations decreased below 2 ng/ml, which is a value necessary to maintain pregnancy, CL function was terminated and all pregnant bitches aborted [36]. This is consistent with findings reported by Onclin et al. [42], i.e., bromocriptine decreased P4 levels below 2 ng/ml in all dogs when applied 40 days after first mating and in approximately 66% dogs (four out of six dogs used in that study) when applied 30 days after mating. This, together with the above-cited shortening of the CL-phase in dogs hypophysectomized on day 4 of the luteal life span, can be seen as indicative of the time-dependent increase in sensitivity of the canine CL towards PRL during progression of the luteal phase. The negative effects of bromocriptine on P4 secretion could be reversed by treatment with PRL but not with LH [43], indicating an indirect effect of bromocriptine on luteal function but also suggesting a superior role of PRL in maintenance of canine CL function compared with LH. This is also consistent with the observation that blocking LH function at the time of full PRL-dependency of the CL resulted in only a transient P4 decrease [36], even though LH was capable of stimulating P4 production *in vivo* [35]. The latter finding is, however, in contrast with observations by Onclin and Verstegen [38] who reported unchanged P4 concentrations after LH application. Consequently, for the mature canine CL, i.e., during the second half of diestrus, PRL becomes the main luteotrophic hormone [36-38]. This is also in contrast to other domestic animal species in which CL function strongly depends on provision of LH, e.g., pigs [44], horses [45] or cattle [46]. Moreover, besides its probable direct effect on lutein cells, LH seems to be capable of stimulating PRL secretion as shown in one study [38], indicating a possible indirect role of LH as a mediator in PRL-dependent P4 synthesis. On the other hand, PRL did not stimulate LH production and suppression of PRL secretion did not affect LH levels [43]. There was also

no direct stimulatory effect of PRL on plasma P4 [43]. Consequently, although PRL appears to be the essential luteotrophic factor from the mid-luteal phase onwards, both during pregnancy and in the non-pregnant canine cycle, its role was suggested in sustaining CL function and/or slowing down luteal regression, rather than in active stimulation of P4 production [43].

5.4. Expression of prolactin- and luteinizing hormone-receptors (PRLR and LHR)

Recently, the partial sequence of canine PRLR cDNA corresponding to the portions of the extra- and trans-membrane domains conserved within the PRLR isoforms of other species was cloned and sequenced in our laboratory [23]. Making use of its availability, the expression of PRLR was investigated in canine CL from non-pregnant and pregnant animals, revealing a time-dependent expression pattern with highest messenger and also protein levels observed at the beginning of the CL phase, and significantly decreased levels towards the end of the luteal life span and/or luteolysis [23]. This cycle-stage related expression of PRLR implies a possible functional interrelationship with respect to circulating P4 levels and suggests that PRLR could act as an upstream regulator in luteal steroid generation in the dog, e.g., at the level of STAR and/or 3βHSD, since their expression patterns resemble that of PRLR. Further studies aimed at delineating complete cDNA of dog-specific PRLR and its potential isoforms are ongoing.

As for the LHR, its expression has not been investigated to date at the molecular level in canine CL. The only contribution is by Fernandes et al. [47] who reported on LHR-binding sites in luteal samples throughout diestrus of non pregnant dogs, which did not change significantly over time. Here, as presented in Fig. 3, the expression of LHR was investigated in luteal samples collected from non-pregnant dogs during the course of diestrus (days 5, 15, 25, 35, 45 and 65 after ovulation; n=5-6 per group), as well as from pregnant dogs during the pre-implantation period (days 8-12, n=5, determined by embryo flushing), post-implantation (days18-25, n=5), midgestation (days 35-40, n=5) and at prepartum luteolysis (n=3). The day of ovulation was determined by monitoring P4 content in peripheral blood, and was defined as the day when P4 levels reached values of 5 ng/ml [9]. For pregnant dogs, the day of mating was recorded (day 0). Prepartum luteolysis was controlled by determining P4 levels every 6 hr beginning with day 58 of pregnancy; when P4 levels continued to decrease below 3 ng/ml within two consecutive measurements, ovario-hysterectomy was performed. More detailed descriptions of the experimental procedure can be found in [14,15,23].

The luteal expression of LHR was investigated at the mRNA level using the in GenBank

available canine-specific partial cDNA sequence. Its expression was strongly time-related ($P<0.01$) in both pregnant and non-pregnant bitches (Fig. 3). In the non-pregnant dogs, the formation of CL was associated with increasing LHR expression, which was significantly ($P<0.001$) upregulated at days 15 and 25 after ovulation. The decrease observed thereafter, towards day 35, was significant ($P<0.05$) compared with the highest expression at day 15. The apparently increased expression on days 45 and 65 was not significant ($P>0.05$). A less dramatic expression pattern was observed for LHR during pregnancy. Its expression increased continuously towards mid-gestation ($p<0.05$), and the apparent decrease during prepartum luteolysis was not significant ($P>0.05$).

The diminished expression of PRLR and LHR in CL of non-pregnant dogs coincides with the onset of luteal regression. Whether this is a trigger or a result of the slowly ongoing degeneration process, leading to reduced P4 synthesis, remains to be elucidated. Together, however, with the observation that PRL did not stimulate P4 synthesis directly, but seemed rather to be involved in sustaining CL function [43], this finding suggests that the PRLR-mediated decline in luteal responsivity to gonadotrophic support could be one of the possible triggers leading to termination of canine CL function. This could also provide an explanation of one of the most intriguing peculiarities of canine reproduction, which is the fact that even if gonadotrophins are needed for proper luteal maintenance, luteal regression/luteolysis take place in spite of continuously increasing availability of PRL and LH, because the function of the degenerating CL cannot be rescued by luteotrophic stimulation.

Even though, based on the above-discussed evidence, the role of LH in regulating CL function in dogs seems to be secondary to that of PRL, the meaning of the deviating expression profile of LHR in the CL of pregnancy merits further investigation, possibly involving more frequent tissue sampling allowing for more thorough temporal analysis of gene expression.

For all semi-quantitative real time (TaqMan) PCR analyses presented herein, our previously described protocols [21,23,48] were applied. Samples were always run in duplicate with three reference genes: GAPDH, 18RrRNA and cyclophilin A, used for normalization of target gene expression in the comparative $\Delta\Delta$CT method. The so-called 'RT minus' controls (samples that were not reverse-transcribed) and 'no-template' controls were included in every experiment. Reactions were set to achieve approximately 100% efficiency. Primers and FAM/TAMRA-labelled TaqMan Probes sequences are listed in Table 1.

6. Intraluteal prostaglandins

Luteal growth involves the upregulated expression of cyclooxygenase 2 (COX2, PTGS2), the rate-limiting factor in the synthesis of prostaglandins (PGs) [14,49]. Its expression correlates positively with the expression of PGE2-synthase (PGES) [14,50] (a downstream enzyme responsible for the conversion of PTGS2-derived PGH2 to PGE2), as well as with the expression of prostaglandin transporter (PGT). The initially elevated expression of both enzymes decreases significantly during luteal regression, as does the expression of PGT, indicating the role of intraluteally produced PGs during the formation but not the regression of the canine CL [2,14,49,50].

Aiming to delineate possible autocrine and paracrine regulatory mechanisms, recently compelling functional evidence has been provided by our group for the luteotrophic function of PGE2 within the canine CL. Prostaglandin E2 proved to be an activator of steroidogenesis in primary luteal cell cultures isolated from the early luteal stage, acting at the level of STAR protein without having a significant impact on the 3βHSD and P450scc enzymatic activity [2]. Expression of the two cAMP-pathway-linked PGE2 receptors, EP2 and EP4 was clearly detectable at the protein level in CL isolated from pregnant dogs. These receptors were localized in lutein cells and revealed CL-stage dependent expression patterns. Whereas the expression of EP2 decreased towards prepartum luteolysis, EP4 expression was biphasic and peaked twice, during the early luteal phase and, unexpectedly, also at mid-gestation [2]. The potential functional importance of these findings, as well as the relative functional involvement of both receptors in luteal maintenance, merits further study, also in context of the possible interplay between PGE2 and other luteotrophic factors, e.g., PRL or LH.

Based on the already mentioned fact that in the dog luteal function is not affected by hysterectomy, a hypothesis was put forward that PGF2α could act locally and intraluteally as a luteolytic factor in the diestrous bitch. Consequently, the luteal expression of PGF2α-synthase (PGFS), classified as AKR1C3, was investigated throughout diestrus [14,51]. AKR1C3 is the only known canine-specific PGFS isoform isolated to date [51] and is responsible for the enzymatic conversion of PGH2 to PGF2α [52,53]. As determined at the mRNA-level, its expression was either absent or very weak, frequently below the detection limit, in the CL of non-pregnant bitches [51]. Together with the low PGF2α-metabolite (13,14-dihydro-15-keto-PGF2α; PGFM) levels observed in peripheral blood during luteal regression [54], this further points towards the lack of an endogenous luteolysin in non-pregnant dogs. Most recently, applying a canine-specific custom-made antibody, PGFS (AKR1C3) expression was assessed at the protein level in the CL of pregnancy revealing

barely detectable signals [52,53]. Interestingly, the PGF2α-receptor seems to be constitutively expressed in the canine CL. It is localized solely in the lutein cells and its mRNA level increases with the progression of the luteal phase, although it is less pronounced during pregnancy [14,51]. The latter might explain the shift in luteal receptivity to exogenously administered PGF2α later in diestrus.

Due to chemical instability and catabolic inactivation, PGs exhibit a very short half-life *in vivo*. The first step in their enzymatic deactivation is the 15-hydroxy prostaglandin dehydrogenase (HPGD)-mediated oxidation of the 15-hydroxyl group to a 15-keto group [55,56]. This process generates the biologically inactive metabolites of PGE2 and PGF2α, PGFM and 13,14-dihydro-15-keto PGE2 (PGEM). Consequently, HPGD is the key enzyme regulating the biological availability of prostaglandins. Making use of the recently cloned canine-specific HPGD cDNA sequence [52,53], its luteal expression was investigated throughout pregnancy and at prepartum luteolysis, revealing strongly individually varying values that tended to be higher at earlier luteal stages and decreasing towards parturition [52].

Here, the luteal expression of HPGD was determined in canine CL obtained from non-pregnant bitches hysterectomized between days 5-65 after ovulation (the same samples as for determining LHR expression) (Fig. 4). For primers and FAM/TAMRA-labelled TaqMan Probe sequences see in Table 1. There was a significant effect of time for luteal HPGD expression in non-pregnant dogs ($P<0.01$, Fig. 4); it was lowest at day 5 after ovulation, then increased significantly, peaking at day 15 ($P<0.01$), with no further significant changes observed during the slow process of luteal regression. The expression pattern of HPGD presented in Fig. 4 seems to be negatively correlated with the previously reported PGES and PGT expression [2,50], implying its role as a factor involved in the local supply of luteotrophic PGE2.

7. Prepartum Luteolysis

Keeping in mind the abrupt P4 decline and the associated massive apoptotic signals detected in the CL during prepartum luteolysis (Fig. 2), occurring concomitantly with strongly elevated PGF2α output, it seems obvious that the prepartum luteolysis is an actively regulated process. Another apparent sign of luteolysis is the significant drop in luteal STAR expression [2,14].

As the vascularization of the CL was already slowed down by the mid-luteal phase and did not change significantly later on, Hoffmann et al. [6,17] postulated that changes in the vascular supply are probably less important as contributors to luteal regression/luteolysis than

in other species. Indeed, in accordance with this hypothesis, in the present experiments (Fig. 5A-C), the mRNA expression of VEGF164 and the two VEGF receptors, VEGFR1 (Flt1) and VEGFR2 (KDR/Flk1), did not differ significantly (P>0.05) between luteal samples obtained from mid-pregnant dogs (days 35-40 of pregnancy; n=5) vs. those collected during prepartum luteolysis (n=3) (Fig. 5A-C). On the other hand, however, in our ongoing study, prepartum luteolysis was associated with significantly increased expression of endothelin receptor type A (EDNRA; ETA), a vasoactive factor known for its strong vasoconstrictor properties. At the cellular level ETA was localized in luteal capillary endothelial cells. No such increase was observed during luteal regression in non-pregnant dogs *(own data, unpublished)*. The functional implication of these findings and their role in the luteolytic cascade remain to be elucidated. In the present experiments, the expression of VEGF system was assessed using primers and TaqMan Probes listed in Table 1.

Because the canine PGFS (AKR1C3) was cloned for the first time using mRNA isolated from the uteroplacental compartment [51], we felt prompted to investigate the capacity of these tissues to produce prostaglandins, with respect to prepartum luteolysis. Consequently, it was concluded that the prepartum increase in luteolytic PGF2α may originate in the upregulated expression of PTGS2 (COX2) within the uteroplacental unit, more specifically, in the *placenta fetalis*, where the upregulated PTGS2 is co-localized with PGFS (AKR1C3) [15]. Our findings agree with the conclusion of Luz et al. [54,57] who postulated the placenta as the main source of the luteolytic PGF2α increase. The underlying alterations in the feto-maternal communication between the maternal stroma-derived decidual cells, the only cells expressing PGR in the canine placenta, and the fetal trophoblast cells, seem to possess a triggering function in the subsequent luteolytic endocrine cascade [15].

8. Antigestagens

As demonstrated in experiments utilizing an antigestagen (aglepristone) in mid-pregnant dogs, interfering with P4 function and/or activity at the level of its receptor initiates an endocrine cascade closely resembling that observed during normal luteolysis. Thus, the uteroplacental prostaglandin system becomes activated, resulting in increased uteroplacental PGF2α output and leading to preterm luteolysis and/or abortion [15,58]. At the CL level, significant downregulation of STAR and 3βHSD is observed and is mirrored in dropping peripheral P4 levels [14]. Similar to normal luteolysis, at least initially at the beginning of the response to antigestagen treatment, the morphological alterations do not involve vascular

changes, as shown by the initially unaltered luteal expression of the VEGF system within the first 24 hrs after the antigestagen treatment (Fig. 5A-C). Functionally, as found in our ongoing study, expression of the vasoconstrictor endothelin receptor A is strongly elevated *(own data, unpublished)*.

9. Conclusions

Amongst the domestic animal species, the dog is the only one with a luteal phase whose duration in non-pregnant animals exceeds that observed during pregnancy. This is not only unique, but also implies distinctly different mechanisms regulating the function of corpora lutea in both situations. In non-pregnant animals, being independent of any uterine luteolysin, the CL fully completes its inherent life span, slowly undergoing morphological and functional changes that, devoid of an active luteolytic principle, appear to be mostly related to the ageing of the CL. This is evidenced by the progressive fatty degeneration of luteal structures. In contrast, in pregnant animals, the initially slow luteal regression seems to be interrupted by the suddenly occurring prepartum luteolysis, associated with strongly increasing peripheral levels of (mostly placenta-derived) PGF2α targeting the constitutively-expressed luteal PGF2α receptor and, thereby, actively destroying the CL (Fig 6).

Several factors seem to regulate luteal function in the dog, presenting an apparent functional shift during different stages of luteal development: whereas prostaglandins seem to be involved rather in CL formation, gonadotrophic support seems to be more important for luteal maintenance (Fig. 7). The modulatory role of many other biologically active factors, e.g., those related to energy balance or glucose uptake, such as insulin receptors and glucose transporters, is implicated and is currently under discussion and/or investigation.

Nevertheless, mechanisms governing canine CL function are still far from being well understood. The impaired response to trophic stimulation of canine luteal cells isolated from later luteal stages [2,19] indicates the presence of other, not yet defined, endocrine and/or paracrine regulatory mechanisms, making the canine CL an experimental model that is difficult to examine, yet one that promises to yield new insights into the biology of this structure.

Conflict of interest: The author has no conflict of interest to declare.

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Figure captions

Table 1

List of primers used for Real Time (TaqMan) PCR.

Figure 1

Histomorphological appearance of canine corpus luteum (CL) at days 5, 25, 45 and 65 post-ovulation (p.o.; hematoxylin and eosin staining). (A,B) early luteal development at day 5 p.o. (corpus hemorrhagicum): extravascular erythrocytes (white arrowheads) can still be observed, distances between cells are relatively large, however, already at this stage well-established capillary networks indicate intense vascularization. (C,D) day 25 p.o., mature CL, reaching maximal steroidogenic capacity; fully developed lutein cells are polyhedral, tightly packed, with homogeneous cytoplasm (white arrows), while the luteal vascular bed is fully developed. (E,F) day 45 p.o., intercellular distances become larger, the density of capillaries decreases, the number of lutein cells also starts to decrease, larger lipid droplets begin to be visible in the cytoplasm of lutein cells (i.e., fatty degeneration becomes evident, black arrows), the number of connective tissue components increases. (G,H) day 65 p.o., marked signs of degeneration are visible in the form of large lipid vacuoles in the cytoplasm of lutein cells (black arrows), which become irregular in shape and size, chromatin condensation commences, pyknotic nuclei can be found, the content of connective tissue elements increases significantly.

Figure 2

Immunohistochemical detection of active caspase-3 in canine CL at the time of prepartum luteolysis, at low and high magnification (white arrows). The white arrowhead in the inset to the right hand picture shows a strongly degenerated lutein cell with large lipid vacuoles and pyknotic nucleus staining strongly for active caspase-3.

Figure 3

Expression of canine LH receptor (LHr) as determined by real time (TaqMan) PCR: (A) CL from non-pregnant cycles, days 5-65 p.o. (B) CL of pregnancy. Numerical data are presented as the mean ± standard deviation (SD). One-way ANOVA (**P<0.05**) was applied followed by the Tukey-Kramer multiple comparison test. Bars with different letters in Fig. 3B differ at $p<0.05$.

Figure 4

Time-dependent expression of canine 15-prostaglandin dehydrogenase (HPGD) as determined by real time (TaqMan) PCR in luteal samples from non-pregnant cycles, days 5-65 after ovulation. Numerical data are presented as the mean ± standard deviation (SD). One-way ANOVA ($P<0.01$) was applied followed by the Tukey-Kramer multiple comparison test. Bars with different letters differ at $p<0.01$.

Figure 5

Expression of vasoactive endothelial growth factor (VEGF) (A), VEGF-receptor 1 (VEGFR1) (B) and VEGF-receptor 2 (VEGFR2) (C) as determined by Real Time (TaqMan) PCR in luteal samples from dogs during mid-gestation and normal prepartum luteolysis (left hand panels), as well as during luteolysis induced with an antigestagen Aglepristone® compared with the mid-gestation group (right hand panels). Numerical data are presented as the mean ± standard deviation (SD). For comparison between the mid-gestation and normal prepartum luteolysis, an unpaired t-test was performed. To evaluate changes in expression of target genes in response to the antigestagen, one-way ANOVA ($P<0.01$) was performed followed by Dunnet's multiple comparison test with the mid-gestation group used as a non-treated control (the respective results present the n-fold change in target gene expression compared to its mRNA-levels at mid gestation). Bars with different letters differ with $P < 0.05$ in Fig. 5A, and $P<0.01$ in Figs. 5B and C.

Figure 6

Schematic presentation of hormonal mechanisms regulating luteal regression in non-pregnant dogs and prepartum luteolysis in pregnant ones, (modified after Kowalewski et al., [3]).

Figure 7

A schematic model illustrating proposed hormonal mechanisms regulating canine corpus luteum (CL) function. The early luteal phase is characterized by intense proliferation (indicated by increased expression of the Ki67 proliferation marker), high vascularization and strongly upregulated expression of steroidogenic acute regulatory protein (STAR) and 3β-hydroxysteroid-dehydorgenase (3βHSD), which is reflected in rapidly increasing peripheral progesterone levels. During this time, the CL displays (more or less pronounced) refractoriness to gonadotrophic support, while the intraluteally-produced prostaglandins are among the important luteotrophic factors. This is reflected in the upregulated expression of cyclooxygenase 2 (COX2), PGE2-synthase (PGES) and prostaglandin transporter (PGT), and downregulated expression of 15-prostaglandin dehydrogenase (HPGD). Additionally, PGE2 was proved to act as a luteotrophic factor in lutein cells from this early luteal phase. Both prolactin (PRL) and luteinizing hormone (LH) are luteotrophic factors, with PRL being the predominant one, especially during the second half of dioestrus when the canine CL is fully gonadotrophin-dependent. As the canine CL passes through its highest steroidogenic activity (about days 15-30 after ovulation), slow luteal regression sets in and the expression of STAR and 3βHSD starts to decrease. At day 30 p.o. smooth endoplasmic reticulum (sER: an organelle in which microsomal enzymes like 3βHSD, P450arom, 17αHSD or inducible PGES isoform, i.e., mPGES, are being synthesized) reveals whirl-like structures and starts to degenerate, which is clearly visible by day 45 after ovulation when it encircles large lipid droplets - a further sign of ongoing fatty degeneration. Peripheral progesterone levels gradually decrease. Even though PRL and LH levels increase during this time, the expression of their respective receptors decreases, likely due to the ongoing degenerative process. Around day 60 of the CL life span, progesterone levels reach the so-called “lower threshold level” [3, 15, 36, 42, 59], which leads to alterations in the placental feto-maternal cross-talk, resulting in activation of the utero-placental prostaglandin system and prepartum PGF2α output (resembling an effect that can be achieved after application of a progesterone receptor blocker = antiprogestin).

Figure 1

Kowalewski

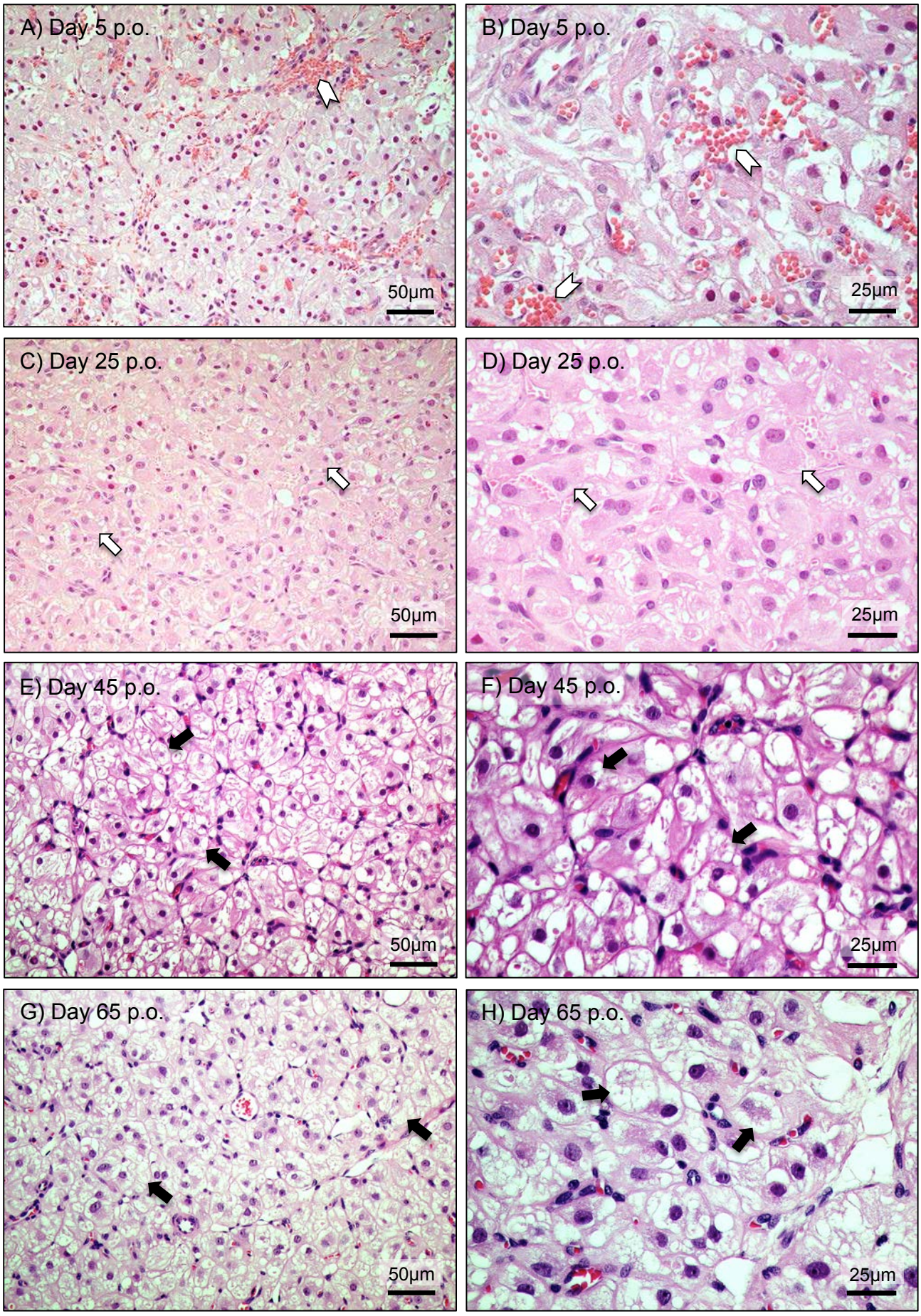

Fig. 1

Figure 2

Kowalewski

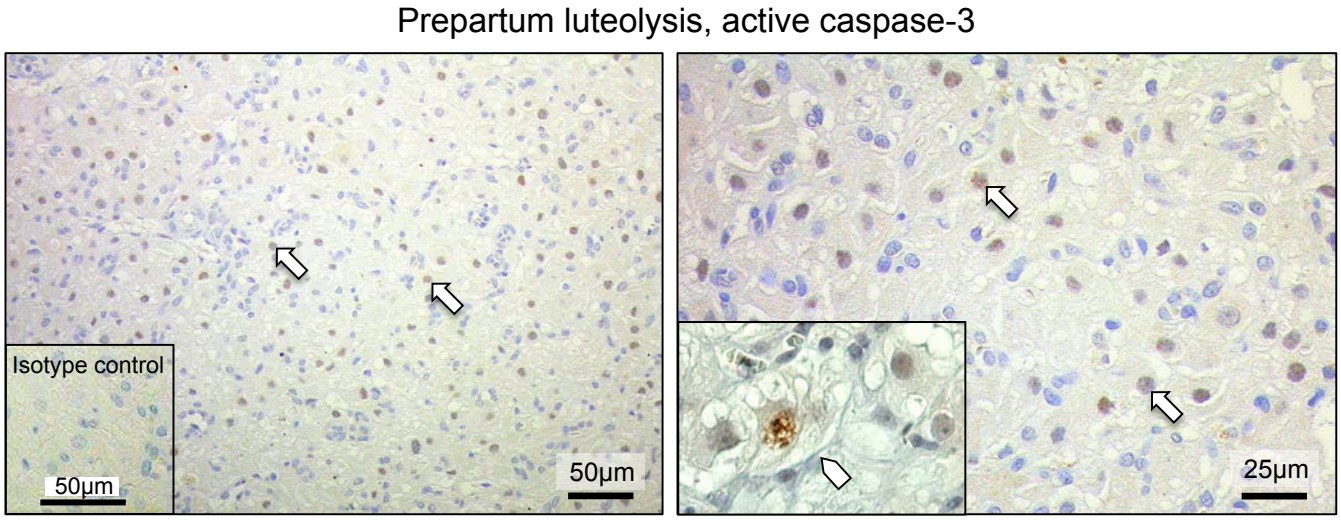

Fig. 2

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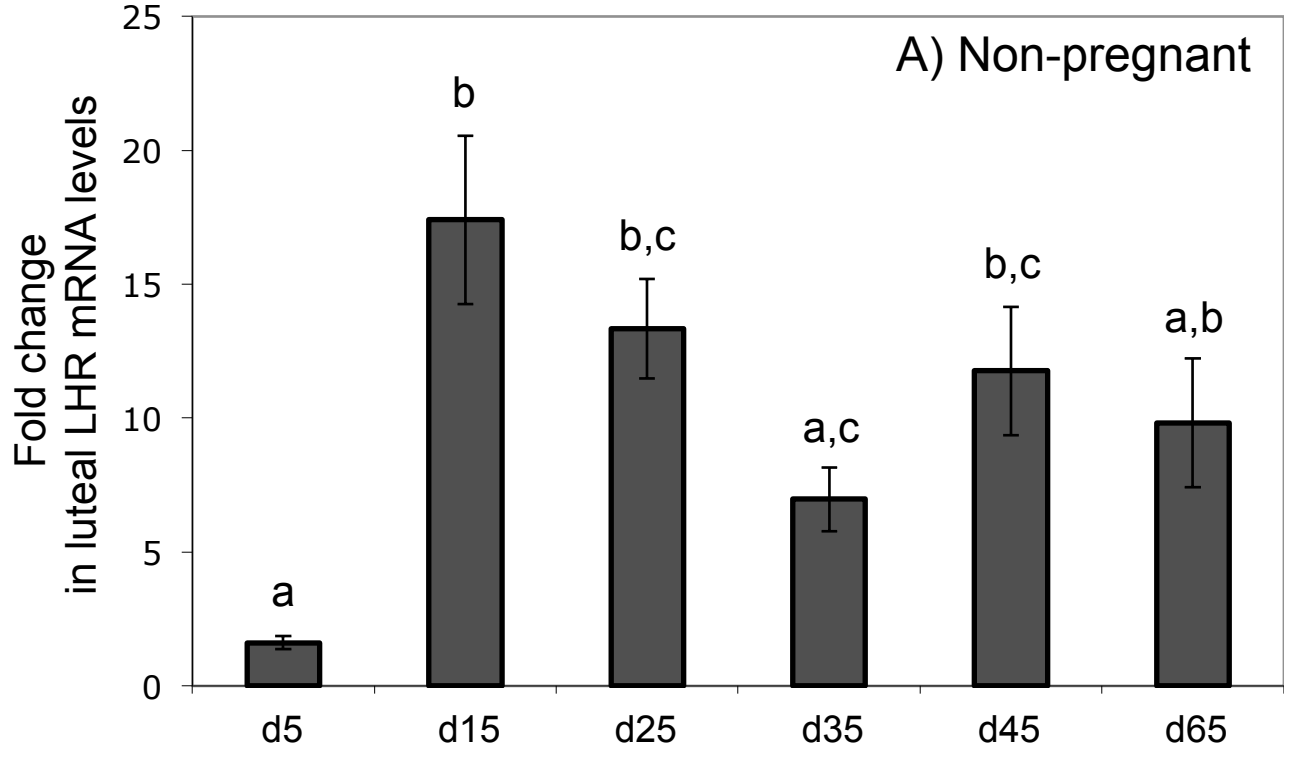

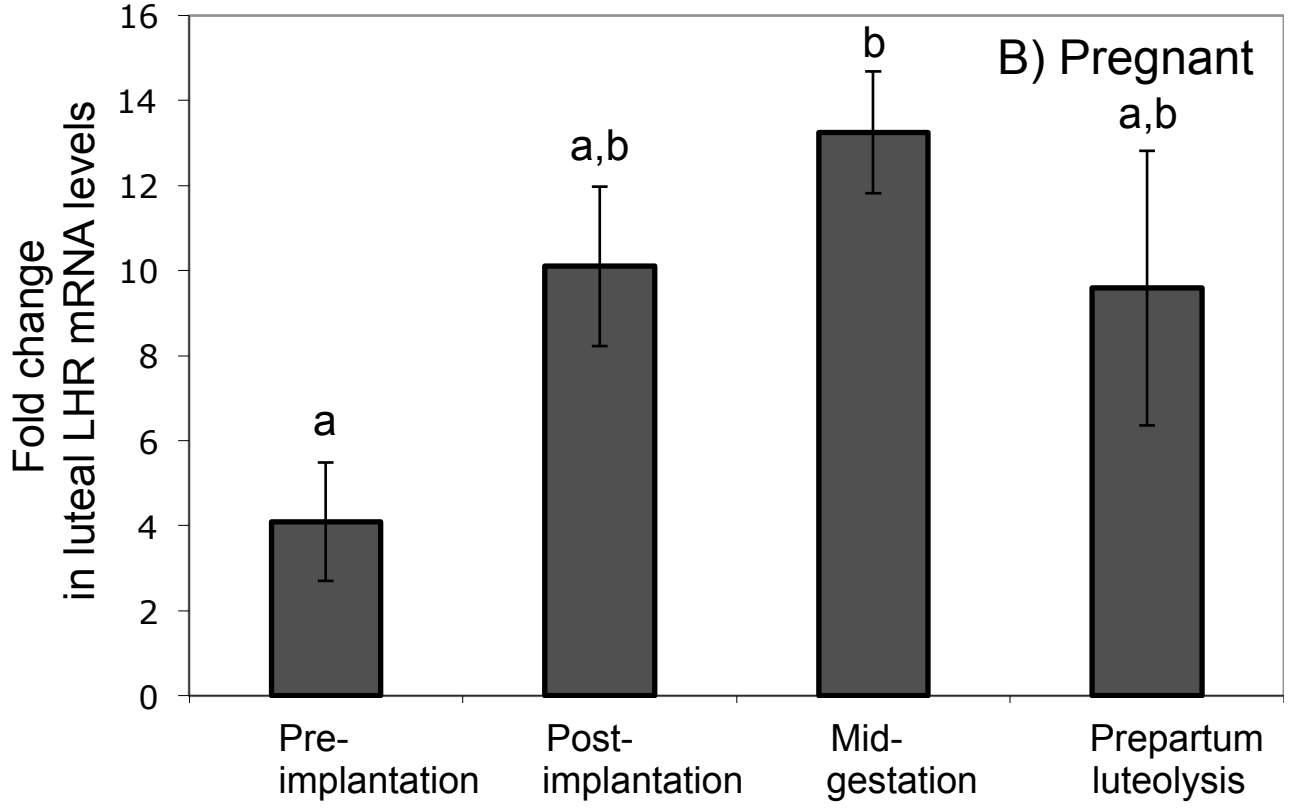

Fig. 3

Figure 4

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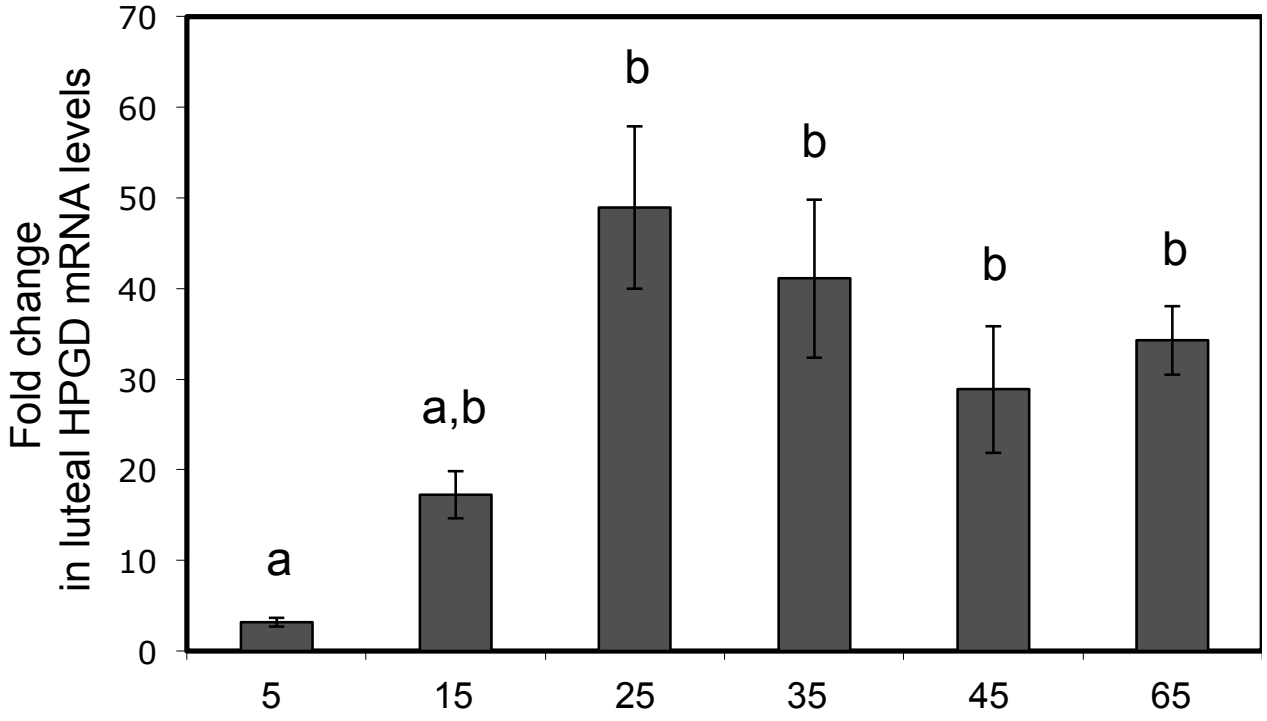

Fig. 4

Figure 5

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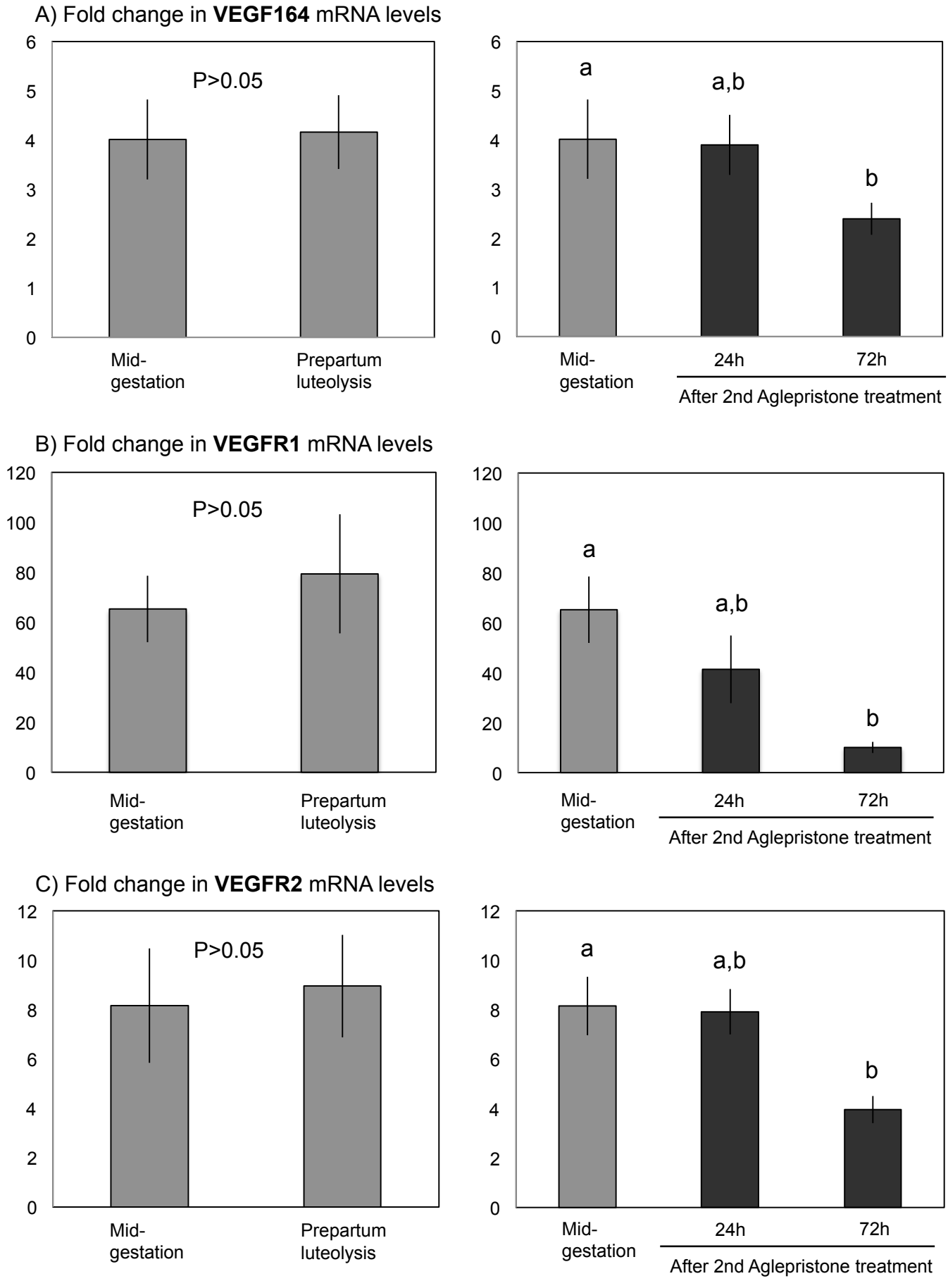

Fig. 5

Figure 6

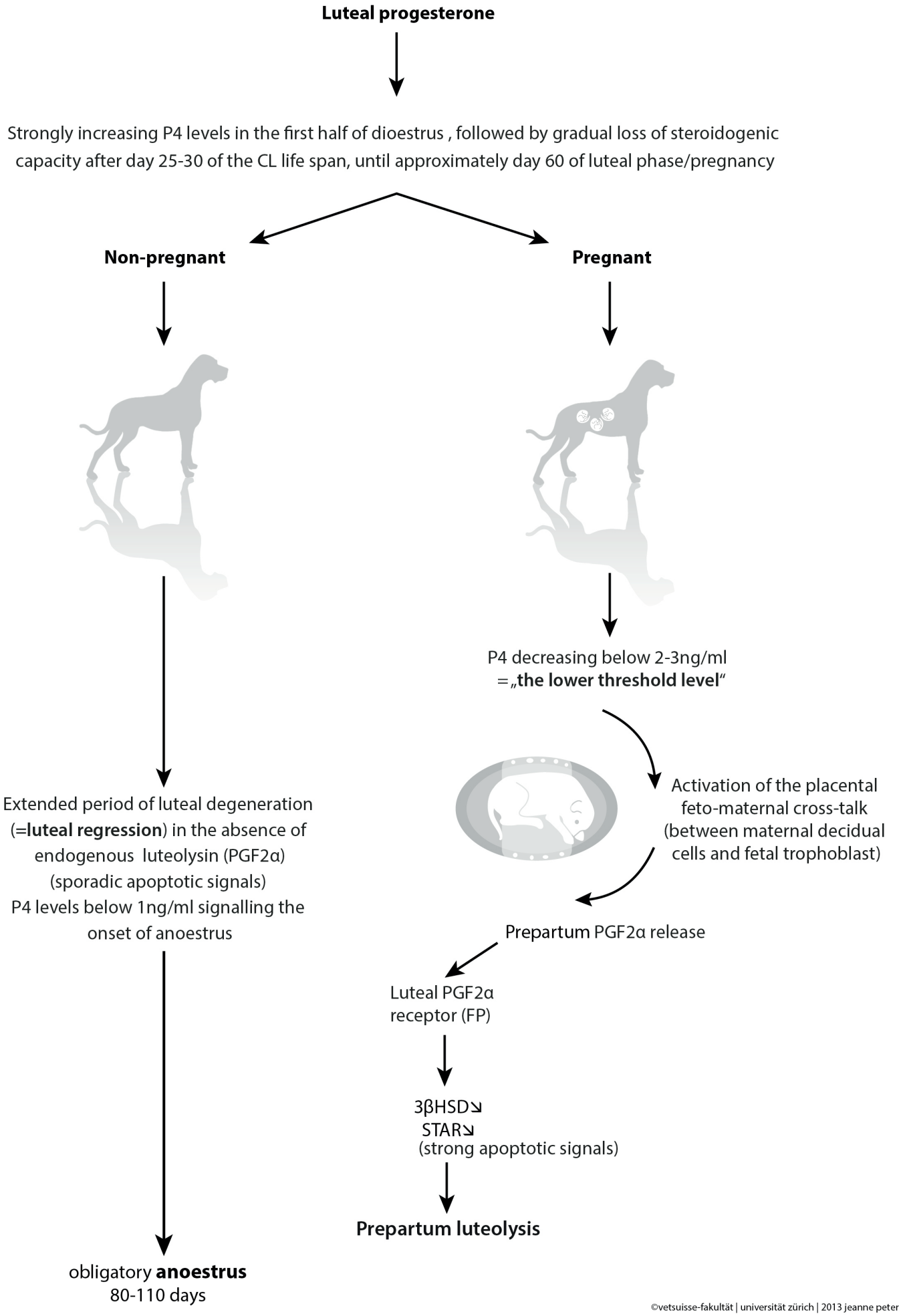
Luteal progesterone
Strongly increasing P4 levels in the first half of dioestrus , followed by gradual loss of steroidogenic capacity after day 25-30 of the CL life span, until approximately day 60 of luteal phase/pregnancy
Non-pregnant
Pregnant
P4 decreasing below 2-3ng/ml
=„the lower threshold level"
Activation of the placental feto-maternal cross-talk (between maternal decidual cells and fetal trophoblast)
Extended period of luteal degeneration (=luteal regression) in the absence of endogenous luteolysin (PGF2α) (sporadic apoptotic signals)
P4 levels below 1ng/ml signalling the onset of anoestrus
Prepartum PGF2α release
Luteal PGF2α receptor (FP)
3βHSD↘
STAR↘
(strong apoptotic signals)
Prepartum luteolysis
obligatory anoestrus
80-110 days
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Figure 7

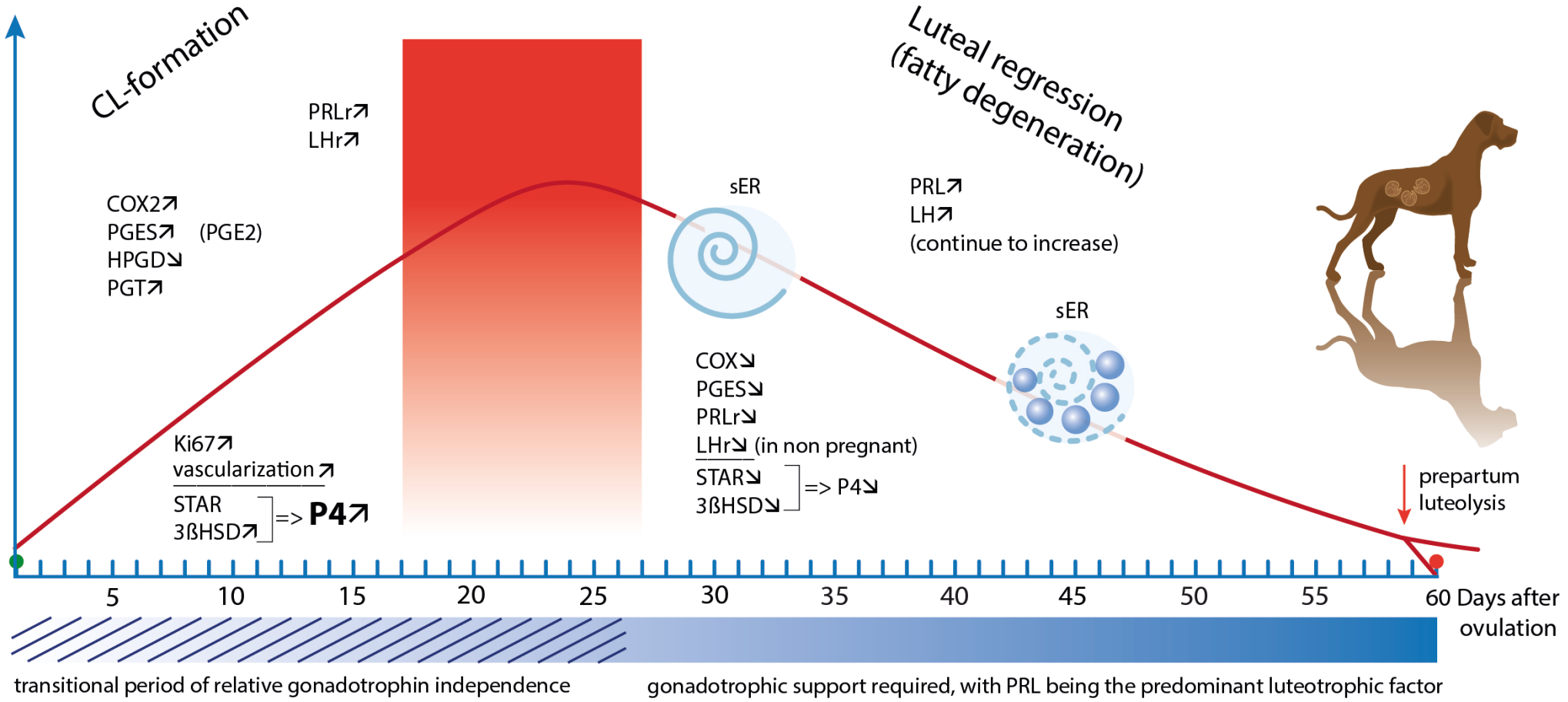
CL-formation
PRLr↗
LHr↗
COX2↗
PGES↗ (PGE2)
HPGD↘
PGT↗
Ki67↗
vascularization↗
STAR
3ßHSD↗
=> P4↗
sER
COX↘
PGES↘
PRLr↘
LHr↘ (in non pregnant)
STAR↘
3ßHSD↘
=> P4↘
Luteal regression
(fatty degeneration)
PRL↗
LH↗
(continue to increase)
sER
prepartum
luteolysis
5
10
15
20
25
30
35
40
45
50
55
60 Days after
ovulation
transitional period of relative gonadotrophin independence
gonadotrophic support required, with PRL being the predominant luteotrophic factor

Table 1

Primer	Accession numbers	Primer sequence	Product length (bp)
LHR-for	AF389885	5’-TCA TCA TTT GTG CTT GCT ACA TTA AA-3’	98
LHR-rev		5’-CGC CAT TTT CTT AGC AAT CTT TG -3’	
LHR-Taq Man Probe		5’-TGC AGT TCA AAA TCC AGA GCT GAT GGC -3’	
HPGD-for	NM_001284477	5’-GGC AGC GAA TCT CAT GAA CAG-3’	93
HPGD-rev		5’-TCT TCT TTC TCA ATG GAT TCA AGG A-3’	
HPGD-Taq Man Probe		5’-TGA ATG CCA TTT GCC CAG GCT TTG T-3’	
VEGF164-for	NM_001003175	5’-GTG CCC ACT GAG GAG TTC AAC-3’	72
VEGF164-rev		5’-CCC TAT GTG CTG GCC TTG AT-3’	
VEGF164-Taq Man Probe		5’-CAC CAT GCA GAT TAT GCG GAT CAA ACC-3’	
VEGFR1-for	AF262963	5’-TGC CTG AAA CAG TGA GAA AGG A-3’	81
VEGFR1-rev		5’-TGC AGA ACT GTT TGC CAT TCC-3’	
VEGFR1-Taq Man Probe		5’-AAA GGC TGA GCA TTA CTA AAT CTG CCT-3’	
VEGFR2-for	DQ269018 / NM_001048024	5’-TGA CAT GGC CTC GGT CAT T-3’	75
VEGFR2-rev		5’-TGT TGG TCG CTA ACA GAA GCA-3’	
VEGFR2-Taq Man Probe		5’-CTA CGT TCA AGA TTA CAG GTC TCC ATT-3’	
GAPDH-for	AB028142	5’-GCT GCC AAA TAT GAC GAC ATC A-3’	75
GAPDH-rev		5’-GTA GCC CAG GAT GCC TTT GAG-3’	
GAPDH-Taq Man Probe		5’-TCC CTC CGA TGC CTG CTT CAC TAC CTT-3’	
18SrRNA-for	FJ797658	5’-GTC GCT CGC TCC TCT CCT ACT-3’	125
18SrRNA-rev		5’-GGC TGA CCG GGT TGG TTT-3’	
18SrRNA-Taq Man Probe		5’-ACA TGC CGA CGG GCG CTG AC-3’	
Cycophilin A	Pre-designed assay from Applied Biosystems, (Foster City, CA, USA) Prod. No. Cf03986523- gH		

***Conflict of Interest Form**

Click here to download Conflict of Interest Form: Kowalewski_Conflict of interest.docx